REMARKS

Formal Matters

Claims 31, 33, 41, and 42 are pending in the application. Claims 34 and 34 are newly canceled without prejudice to later prosecution. Claims 31, 33, 41, and 42 are amended. Support for the amendments is found throughout the specification such as at, for example, page 10, lines 25-27; page 11, line 19 to page 12, line 2 (see page 11, lines 20-21 and page 12, lines 1-2); page 36, lines 30-34; page 66, lines 8-11 and lines 14-19; page 72, line 22 to page 73, line 9; page 73, lines 10-16; page 76, lines 10-17; page 89, lines 1-16; and page 107 line 25 to page 108, line 26 (Example IV). The amendments to claims 33 and 41 are made merely to provide proper claim dependency. The amendments to claims 31, 33, 41, and 42 are made merely to better present the issues on appeal, should an appeal become necessary. No new matter is added by the amendments and the Examiner is respectfully requested to enter them.

Compliance with 37 CFR § 1.821 (a)(2)(d)

The specification is amended to indicate SEQ ID NOs referred to in the Figure recited in the text. Support for the amendments is found throughout the specification such as at, for example, page 8, lines 5-31 and in the Sequence Listing submitted January 18, 2002. No new matter is added by the amendments and the Examiner is respectfully requested to enter them.

Withdrawn rejections

Applicants gratefully acknowledge withdrawal of the rejection of claim 41 under 35 U.S.C. § 112, second paragraph and withdrawal of the rejection of claims 33 and 38-40 under 35 U.S.C. § 112, first paragraph.

Rejection Under 35 U.S.C. § 112, First Paragraph

Claims 31-34 and 41-42 are rejected under 35 U.S.C. § 112, first paragraph, because, allegedly, the specification is not enabling for the claim scope. Claims 32 and 34 are canceled, rendering moot their rejection. Applicants respectfully traverse the rejection as applied and as it might be applied to the currently pending claims for the reasons provided below.

Applicants disclose that CHF (same as CT-1) is useful for maintaining motor neurons and to induce motor neuron sprouting *in vivo* (see the specification at page 74, lines 30-32) and for treating neurodegenerative diseases (specification page 75, lines 3-7). They further disclose useful methods of administration, including injection or infusion systemically (e.g., intravenous injection) or directly (e.g., intralesional) or by sustained-release systems (see the specification at page 76, lines 10-15). In addition, they disclose how to determine an effective amount of CT-1 for administration (see the specification at page 77, lines 21-31).

The Examiner suggests (at page 4 of the Office Action) that the state of the art for treating neurons with CT-1 *in vivo* is as indicated by M. Sendtner in Barinaga, M., Science 264:772-774 (May 6, 1994) published after the priority date of the present application. Applicants respectfully disagree. While the Examiner points out that CNTF

is reported by Sendtner to have a short half-life in the bloodstream of a rat (see page 773, col. 1, third full paragraph), Sendtner goes on to suggest that direct delivery to the cerebrospinal fluid may be an appropriate route of administration to motor neurons (see page 773, same paragraph). Further, Pennica et al. (Neuron 17:63-74 (1996), copy enclosed herewith) report in an article published after the priority date of the present application that CT-1 administered directly to a sectioned sciatic nerve of a rat neonate had a survival-promoting, neurotrophic effect on the motoneurons *in vivo* (see, for example, abstract and page 67, col. 1). This report is consistent with Applicants' disclosure in the specification that direct (e.g., intralesional) administration is an effective route of administration. Thus, Applicants' disclosure in the specification that direct administration of CT-1 to motor neurons is potentially effective is consistent with statements in the literature.

The Examiner also expresses concern (at page 4 of the Office Action) that undue experimentation would be required to determine whether the claimed invention has been successfully practiced. Applicants respectfully disagree. As stated above, Applicants disclose useful methods of directly administering CT-1 to promote survival of motor neurons, which disclosure is consistent with subsequently published literature. Such disclosure, coupled with ordinary skill in the art, is sufficient to practice a method of increasing survival of a motor neuron by administering a polypeptide that exhibiting neurotrophin activity and having an amino acid sequence identity of at least 70% to SEQ ID NO:3 or 8, but not rat CT-1.

The Examiner further suggests, based on the teaching of Rudinger (previously of record) that CT-1 variants of SEQ ID NO:3 or 8 would be predicted to have no survival-

promoting activity. Applicants respectfully disagree. First, not all variants of a given polypeptide are expected to be inactive. Some variants of the CT-1 of the claim would reasonably be expected to have the function of neurotrophin activity for a motor neuron. Variants can be readily tested for the claimed function of neurotrophin activity according to the CNTF *in vitro* neurotrophic activity assay disclosed by Applicants in the specification (for example, at page 72, line 22 to page 73, line 9 and at page 107, line 24 to page 108, line 26 (Example IV)). Further, Pennica et al. (Neuron, 1996, *supra*) showed that CT-1 is effective *in vivo*, consistent with Applicants disclosure. Thus, one of ordinary skill in the art is readily able to test variants for the function of neurotrophin activity and, as a result, the metes and bounds of the claims with respect to the CT-1 variant is readily determined according to Applicants' disclosure.

The rejection under 35 U.S.C. § 112, first paragraph, having been overcome, Applicants respectfully request reconsideration and allowance of the claims.

Rejection Under 35 U.S.C. § 112, Second Paragraph

Claim 34 is rejected under 35 U.S.C. § 112, second paragraph, as allegedly being improperly dependent from claim 32. Applicants respectfully traverse the rejection as applied and as it might be applied to the currently pending claims for the reasons provided below.

Claim 34 is amended to depend from claim 31. Withdrawal of the rejection and allowance of the claim is respectfully requested.

SUMMARY

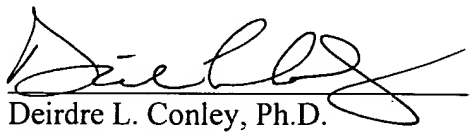
Claims 31, 33, 41, and 42 are pending in the application. Claims 32 and 34 are newly canceled. Amendments to the specification and claims are made without prejudice to later prosecution of any subject matter disclosed in the specification and claims. Amendments are intended merely to put the application in order for allowance or to better present the issues for appeal, should an appeal become necessary.

Rejection of the claims under Sections 112, first and second paragraph have been overcome and allowance of the claims is respectfully requested. Applicants respectfully request that a timely Notice of Allowance be issued in this case.

If in the opinion of the Examiner, a **telephone conference** would expedite the prosecution of the subject application, the Examiner is **strongly encouraged** to call the undersigned at the number indicated below.

This response/amendment is submitted with a transmittal letter, Notice of Appeal, article by Pennica et al. (Neuron 1996), and petition for a three-month extension of time and fees. In the unlikely event that this document is separated from the transmittal letter, applicants petition the Commissioner to authorize charging our Deposit Account 07-0630 for any fees required or credits due and any extensions of time necessary to maintain the pendency of this application.

Respectfully submitted,
GENENTECH, INC.

Date: November 24, 2003 By: 

Deirdre L. Conley, Ph.D.
Reg. No. 36,487
Telephone No. (650) 225-2066

Clean Set of All Pending Claims

November 24, 2003

Claims 1-30 (canceled)

31. (Twice Amended) A method of increasing survival of a motor neuron, comprising administering directly to a motor neuron an isolated polypeptide exhibiting neurotrophic activity and having an amino acid sequence identity of at least 70% to SEQ ID NO: 3 or SEQ ID NO:8, but not a rat cardiotrophin-1.

32. (canceled) Please cancel claim 32 without prejudice to later prosecution.

33. (Amended) The method of claim 31, wherein the motor neuron is a ciliary ganglion.

34. (Canceled) Please cancel claim 34 without prejudice to later prosecution.

Claims 35-40 (canceled)

41. (Twice Amended) The method of claim 31 further comprising administering a second neurotrophic factor for increasing neuronal survival.

42. (Twice Amended) The method of claim 41, wherein the second neurotrophic factor is selected from the group consisting of IGF-1, CNTF, NGF, BDNF, NT-3, and NT-4.

Neuron, Vol. 17, 63–74, July, 1996, Copyright ©1996 by Cell Press

Cardiotrophin-1, a Cytokine Present in Embryonic Muscle, Supports Long-Term Survival of Spinal Motoneurons

D. Pennica,* V. Arce,† T. A. Swanson,* R. Vejsada,‡ R. A. P llock,† M. Armanini,§ K. Dudley,† H. S. Phillips,§ A. Ros nthal,§ A. C. Kato,‡ and C. E. Henderson†
*Department of Molecular Biology
§Department of Neuroscience
Genentech Incorporated
South San Francisco, California 94080
†INSERM Unité 382
IBDM (CNRS–INSERM–Université de la Méditerranée)
Campus de Luminy
13288 Marseille Cedex 09
Franc
‡Division of Clinical Neuromuscular Research
and Department of Pharmacology
Centre Médical Universitaire
1211 Geneva 4
Switzerland

Summary

Th muscle-derived factors required for survival of embryonic motoneurons are not clearly identified. Cardiotr phin-1 (CT-1), a cytokine related to ciliary neurotrophic factor (CNTF), is expressed at high levels in embryonic limb bud and is secreted by differentiated myotubes. In vitro, CT-1 kept 43% of purified E14 rat motoneurons alive for 2 weeks (EC_{50} = 20 pM). In vivo, CT-1 protected neonatal sciatic motoneurons against th effects of axotomy. CT-1 action on motoneurons was inhibited by phosphatidylinositol-specific phosph lipase C (PIPLC), suggesting that CT-1 may act through a GPI-linked component. Since no binding of CT-1 to CNTFRα was detected, CT-1 may use a novel cytokine r ceptor α subunit. CT-1 may be important in normal motoneuron development and as a potential tool for slowing motoneuron degeneration in human diseas s.

Introducti n

Peripherally derived neurotrophic factors play a vital role in regulating the survival of spinal motoneurons at all stages of their development. When a limb is removed from a chick embryo before motor axons leave the spinal cord, all motoneurons destined to innervate that limb subsequently die during the normal cell death period (Oppenheim, 1991; Hamburger, 1992). Axotomy of neonatal motoneurons in the spinal cord or brain stem results in rapid death of all affected neurons (Sendtner et al., 1990; Vejsada et al., 1995). In adult animals, axotomy alone does not induce rapid motoneuron death. How-ver, complete interruption of interactions with the periphery by ventral root avulsion leads to loss of the operated motoneurons over the following 6 weeks (Li et al., 1995a). The simplest explanation for th se observations is that cells in th periphery that interact with the motoneuron (and in particular skeletal muscle) produce neurotrophic factors that are required for motoneuron survival throughout the life of the organism.

As a first step toward identifying the factors involved, different in vitro and in vivo systems have been developed in which motoneuron death can be reduced or prevented by the administration of exogenous factors (for review, see Henderson et al., 1993a; Henderson, 1996). Molecules thus identified are candidate physiological motoneuron survival factors and are also potential therapeutic agents for slowing motoneuron death in human diseases such as amyotrophic lateral sclerosis (ALS) and the spinal muscular atrophies (SMA) (Henderson, 1995).

Among the best-studied factors with neurotrophic activity for motoneurons in a variety of experimental situations are the neurotrophins brain-derived neurotrophic factor (BDNF), neurotrophin 3 (NT-3), and neurotrophin 4/5 (NT-4/5) (Oppenheim et al., 1992; Yan et al., 1992; Sendtner et al., 1992b; Henderson et al., 1993b; Vejsada et al., 1995) and a transforming growth factor β family member, glial cell line–derived neurotrophic factor (GDNF) (Henderson et al., 1994; Oppenheim et al., 1995; Yan et al., 1995). They are all expressed in the peripheral environment of the motoneuron during cell death (Henderson et al., 1993b, 1994). Moreover, motoneurons express high affinity receptors for BDNF, NT-3, and NT-4/5 throughout life (Henderson et al., 1993b). Nevertheless, definitive evidence for physiological involvement of these factors in supporting motoneuron survival is currently lacking. In the case of GDNF, no data are available concerning the nature of the GDNF receptor or the phenotype of null-mutant mice. For the neurotrophins and their Trk receptors, no major loss of motoneurons is observed in either single or double knockout mice, at least on the C57/Bl6 genetic background that has been most thoroughly studied (see references in Henderson, 1996), suggesting that neurotrophins may play a role in regulating motoneuron synaptogenesis or function rather than survival.

The interleukin-6 (IL-6) family of cytokines are a group of structurally related polypeptides including interleukin-11 and oncostatin M that mediate their biological effects through a closely linked family of receptors (Davis et al., 1993). Two members of this family, ciliary neurotrophic factor (CNTF) and leukemia inhibitory factor (LIF), keep motoneurons alive when applied exogenously in vitro and in vivo (Arakawa et al., 1990; Oppenheim et al., 1991; Martinou et al., 1992; Sendtner et al., 1992a; Henderson et al., 1994; Sagot et al., 1995; Vejsada et al., 1995), and their receptors are known to be synthesized by motoneurons (Ip et al., 1993; Li et al., 1995b). Nevertheless, their physiological role in embryonic motoneuron survival has been thought to be minimal, in part because levels of CNTF are extremely low before birth (Stöckli et al., 1991; Ip et al., 1993), and in part because mice in which the genes for CNTF, LIF, or both are inactivated do not show significant motoneuron loss during embryogenesis (Stewart et al., 1992; Escary et al., 1993; Masu et al., 1993; Sendtner et al., 1996).

Targeted disruption of gp130, a shared component of the CNTF receptor (CNTFR) and LIF receptor (LIFR)

complexes, leads to myocardial disorders, but the neuronal phenotype is unknown (Yoshida t al., 1996). Recently, however, it has been reported that knockout mice for either the α subunit of the CNTFR (DeChiara et al., 1995) or the β subunit of the transmembrane LIFR (common to both receptor complexes) (Li et al., 1995b) die shortly after birth. In contrast with null mutants for the corresponding factors, both receptor mutants show a loss of approximately 40% of motoneurons in spinal cord and brain stem. These results suggest that an unidentified ligand of the CNTFR and/or LIFR plays a physiological role in regulating motoneuron survival in vivo.

Recently, a novel member of the IL-6 family of cytokines, called cardiotrophin-1 (CT-1), was identified and cloned based on its ability to induce hypertrophy in cultured neonatal cardiac myocytes (Pennica et al., 1995a). In the presence of gp130, CT-1 binds to the LIFRβ subunit with an affinity in the nanomolar range, in a competitive manner with LIF (Pennica et al., 1995b). Lik CNTF, CT-1 has no signal peptide but is secreted by transfected cells in vitro (Pennica et al., 1995a). In addition to its hypertrophic activity, it also enhances survival and maturation of different neuronal populations in culture (Pennica et al., 1995b). Here, we show that CT-1 can provide long-term support to a fraction of motoneurons very similar to that lost in the CNTFR and LIFR knockout mice and that its expression pattern is consistent with a role as a target-derived neurotrophic factor during normal motoneuron development.

Results

Cardiotr phin-1 mRNA Is Present in the Envir nment of the Embryonic Motoneuron

To determine whether cardiotrophin-1 might be available to developing motoneurons, CT-1 mRNA expression was analyzed by in situ hybridization during the period of motoneuron cell death. At embryonic day 13.5 (E13.5) in the mouse, the stage at which motoneuron death is about to begin, there was a strong signal for CT-1 mRNA in several peripheral tissues, with particularly high concentrations in developing limb bud (Figure 1A). Only weak hybridization to spinal cord and other central nervous system structures was observed. No signal was observed when an adjacent section was hybridized with a sense probe (data not shown). At E15.5, a similar pattern was observed, together with strong labeling of bone structures (Figure 1B). After the period of motoneuron cell death, on postnatal day 1 (PN1), no specific signal could be detected on any structure in the limb bud by in situ hybridization (data not shown). In parallel xperiments using a probe for mouse LIF, no specific signal was detected at either E13.5 or PN1 (data not shown).

We used semiquantitative reverse transcriptase–polymerase chain reaction (RT–PCR) to compare levels of CT-1 in limb bud at the beginning and the end of the motoneuron c ll death period. Samples of cDNA prepared from total limb bud of E14.5 mouse embryos and PN1 mouse pups were normalized for GAPDH mRNA (Figure 1C). When RT–PCR was performed on the same sampl s using primers specific for CT-1 (Figure 1C), the amplified fragment (281 bp) hybridized to a specific internal oligonucleotide (data not shown). A strong CT-1 signal was obtained using E14.5 limb bud (Figure 1C). However, in agreement with our results from the in situ hybridization, levels of CT-1 mRNA were markedly reduced by the end of cell death (Figure 1C).

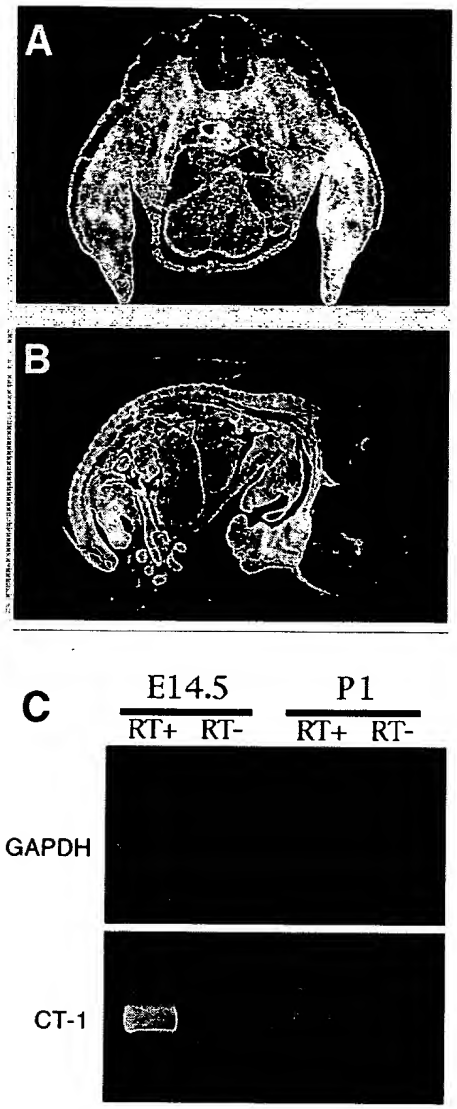


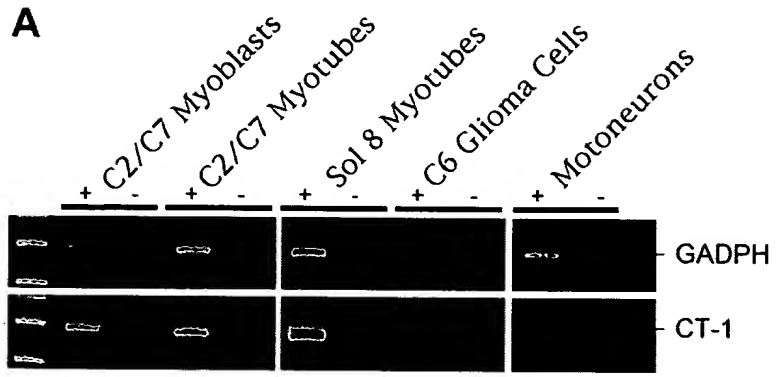
Figure 1. Levels of CT-1 mRNA Are High in Embryonic Limb Bud but Decrease during the Period of Naturally Occurring Motoneuron Death

(A) Cross section of an E13.5 mouse embryo after in situ hybridization for CT-1 mRNA. Note the strong signal in distal limb bud.

(B) In situ hybridization on a longitudinal section of an E15.5 embryo. Only low levels of CT-1 mRNA are apparent in the central nervous system.

(C) Semiquantitative RT–PCR to compare levels of CT-1 mRNA in limb bud at E14.5 and PN1. Amplified fragments obtained by PCR using primers for GAPDH or CT-1 on mRNA samples incubated with (RT+) or without (RT−) reverse transcriptase were stained with EtBr.

These results suggest that CT-1 may be available to motoneurons at high levels as competition for muscle-derived trophic support begins, and that subsequently only lower levels of this factor may be present.



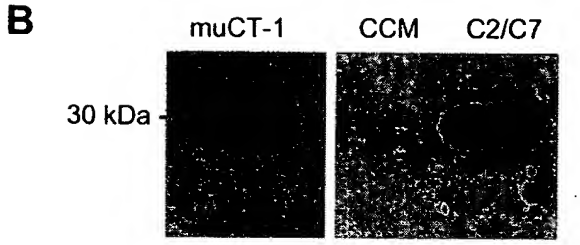


Figure 2. Muscle Cells Express mRNA for CT-1 and Secrete Mature CT-1 Protein

(A) Total RNA from the indicated cells was reverse transcribed and amplified using specific primer pairs for mouse CT-1. All muscle cells expressed high levels of CT-1 mRNA, whereas neither C6 glioma nor purified motoneurons had detectable levels. A plus or minus sign indicates with or without reverse transcriptase, respectively.

(B) Western blots of recombinant mouse CT-1 (CT-1), complete culture medium (CCM), and medium conditioned by C2/C7 myotubes (C2/C7) stained using an affinity-purified antibody to CT-1. Specific immunoreactivity was only observed at an apparent molecular mass of 30 kDa.

Cardiotrophin-1 Is Synthesized and Secreted by Differentiated Myotubes

The high levels of expression in several different peripheral tissues suggested that the target tissue of motoneurons, skeletal muscle, was a potential source of CT-1. We therefore used RT–PCR to detect synthesis of CT-1 mRNA by immortalized muscle cell lines (primary myotube cultures from rodent are contaminated by Schwann cells and fibroblasts and so were not used). Cultures of undifferentiated myoblasts and differentiated myotubes were prepared from three different muscle cell lines: C2/C7 (Catala et al., 1995), Sol8 (Pinset et al., 1991), and 129CB3 (Pinçon-Raymond et al., 1991; data not shown). CT-1 mRNA was detected in all muscle sources, but was absent from C6 glioma cells, used as a control (Figure 2A). Furthermore, in agreement with our in situ hybridization results, no CT-1 mRNA could be detected in purified motoneurons (Figure 2A).

Like CNTF, CT-1 does not have a consensus signal peptide. To confirm that CT-1 could nevertheless be released by muscle cells into their environment, we performed Western blots on samples of conditioned medium from differentiated C2/C7 myotubes. Purified recombinant muCT-1 produced in human 293 cells (derived from embryonic kidney) migrated with an apparent molecular weight of 30 kDa, corresponding to a glycosylated form of the 22 kDa polypeptide (Figure 2B). Using affinity-purified rabbit polyclonal antibodies to muCT-1, we detected a band at 30 kDa in medium conditioned by C2/C7 myotubes, but not in unconditioned medium (Figure 2B). By comparing the intensity of the band on the Western blot with that obtained using different quantities of recombinant CT-1 (data not shown), we estimat that myotube-conditioned medium contains approximately 1–5 ng/ml CT-1. Thus, CT-1 is secreted at biologically significant levels by healthy differentiated myotubes in culture.

CT-1 Is a Potent Long-Term Survival Factor for a Fraction of Purified Motoneurons

Using the culture system for purified motoneurons we previously described (Henderson et al., 1995), we tested the ability of CT-1 to enhance motoneuron survival over 3 days in culture. Like CNTF and LIF (Henderson et al., 1994), 10 ng/ml CT-1 supported ~50% of motoneurons that initially developed in culture (data not shown). To better analyze the trophic effects of CT-1, we developed a new long-term culture system for these highly purified motoneurons, based on the use of Neurobasal medium with the B27 supplement (see Experimental Procedures). All neurons analyzed in this study were large, multipolar neurons that expressed Islet-1/2, an independent marker for spinal motoneurons (Henderson et al., 1994; Tsuchida et al., 1994).

The potency of CT-1 action was determined by direct counting of motoneuron survival in the presence of increasing concentrations of CT-1; a dose-response curve after 6 days of culture in Neurobasal medium is shown in Figure 3. The mean value for the EC_{50} was 550 pg/ml, or 2×10^{-11} M, as calculated from three different experiments in which survival times ranged from 3 to 10 days. This is similar to the values we have reported for rat CNTF in short-term culture (Martinou et al., 1992; Henderson et al., 1994).

Using the possibilities for long-term analysis provided by Neurobasal medium, we cultured motoneurons in the presence and absence of CT-1 for periods up to 16 days in vitro. Even at low density, motoneurons survived and matured in these conditions to a remarkable degree. In the presence of CT-1, motoneurons developed rapidly in culture and after 3 days had developed long axons and multipolar morphology (Figure 4A). After longer periods in the presence of CT-1, morphological development of motoneurons was ven more pronounced. At 9–11 days of culture, surviving neurons showed a highly

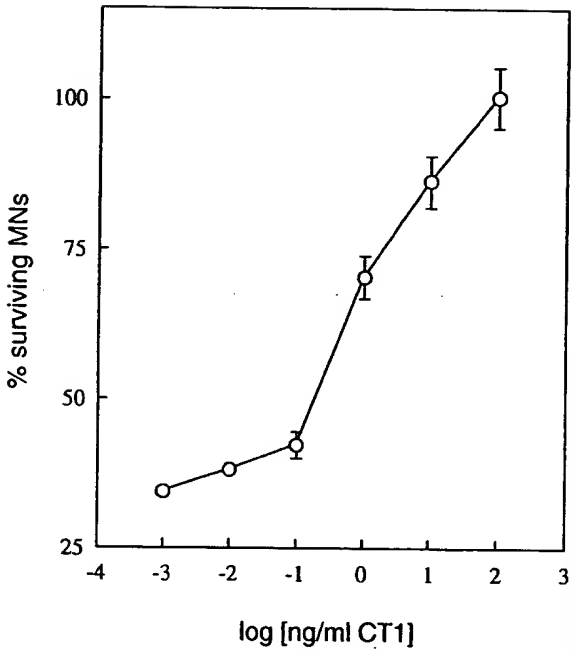


Figure 3. Dose-Response Curve for the Survival-Promoting Effect of CT-1 on Purified E14 Motoneurons after 6 Days in Culture

Values are expressed relative to the number of motoneurons surviving in 100 pg/ml GDNF, determined after 16 hr of culture and taken as 100%. Mean values (±SEM) for triplicate dishes are shown. Calculated EC_{50} for this curve was 450 pg/ml.

multipolar morphology (Figure 4B), with axon-like processes often several millimeters in length (Figure 4C) and tapering, thick dendrite-like processes with many secondary branches (Figure 4D). The theoretical age of E14 motoneurons cultured for 11 days was postnatal day 4; their morphology suggests that many aspects of their maturation occurred normally in culture in the presence of CT-1.

Despite the obviously healthy appearance of surviving motoneurons, optimal concentrations of CT-1 were not capable of maintaining all motoneurons in the long term. In six independent experiments counted between 9 days and 16 days of culture, the fraction of motoneurons surviving in the presence of CT-1 was 43% ± 1%. The corresponding value for cultures without trophic factor was 6% ± 2% (n = 6). In the same experiments, GDNF, the most potent survival factor for motoneurons in short-term culture (Henderson et al., 1994), maintained only 24% ± 6% (n = 6) of motoneurons that initially developed in culture. The number of surviving motoneurons was not increased when different concentrations of CT-1 or GDNF, or different regimens for medium replenishment, were tested (data not shown). These values therefor seem to represent characteristic fractions of motoneurons kept alive by single neurotrophic factors.

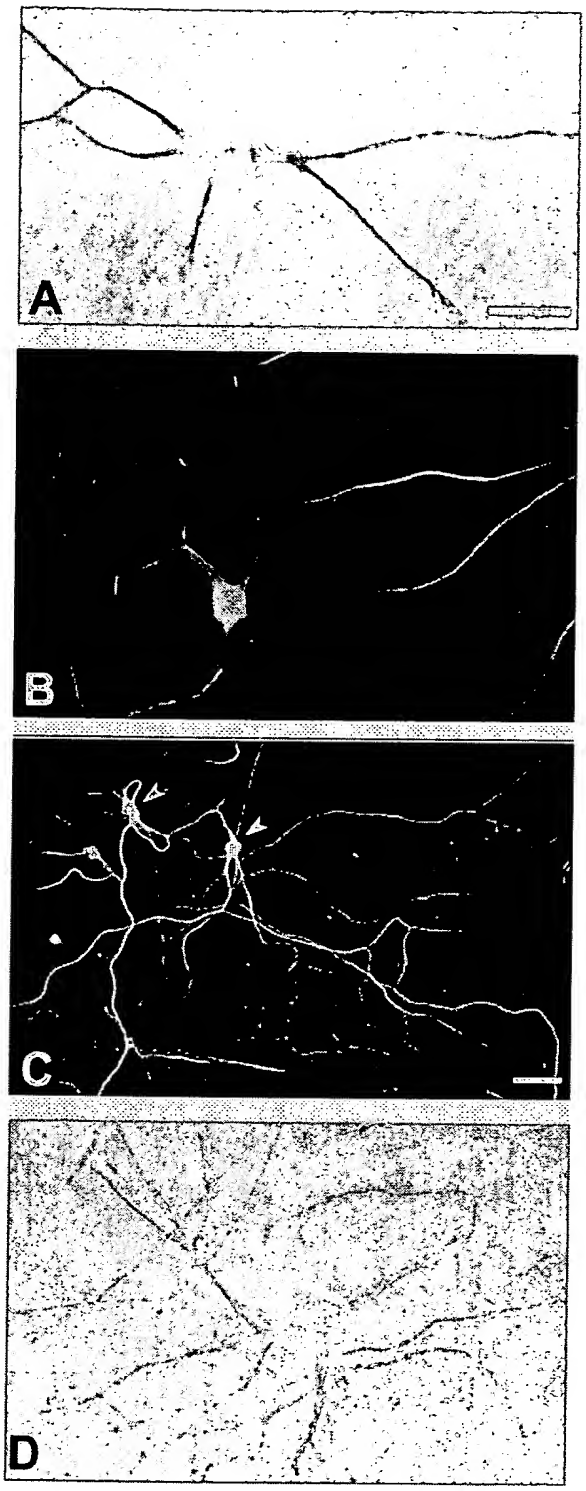


Figure 4. Purified E14 Motoneurons Grown in the Presence of 10 ng/ml CT-1 for Different Periods

(A) Motoneuron after 3 days in culture. Note multipolar morphology, with limited branching.

(B) Motoneuron cultured for 9 days with CT-1, and stained for neurofilament. Each motoneuron has several primary neurites and increased branching.

(C) Low power view of same culture as (B). Only 2 cell bodies are visible in this field (arrowheads). Note extensive neurite network.

(D) Motoneuron cultured for 11 days in CT-1. The asymmetric soma and thick tapering dendrites with many smaller processes are typical of motoneurons in these long-term cultures. Scale bars: shown in (A), 50 μm for (A), (B), and (D); shown in (C), 100 μm for (C).

We compared the capacity of three cytokines of the IL-6 subfamily, CT-1, CNTF, and LIF, to support long-term survival. In 14 experiments in which identical concentrations (10 or 100 ng/ml, depending on the experiment) of mouse CT-1, rat CNTF, or mouse LIF were assayed in parallel, the numbers of motoneurons kept alive by each factor, expressed relative to CT-1, were 100% (CT-1), 93% ± 6% (CNTF), and 80% ± 6% (LIF). When optimal concentrations of different pairs of cytokines were assayed in combination, the number of surviving motoneurons did not increase (data not shown). Therefore, many of the same motoneurons that respond to CT-1 are also kept alive in culture by CNTF or LIF.

Our in vitro results therefore suggest that a characteristic fraction (~45%) of motoneurons survive and mature in the presence of cytokines, in particular the muscle-derived cytokine CT-1. Interestingly, CT-1 alone is 80% more efficacious than GDNF in this system.

CT-1 Protects Motoneurons against the Effects of Neonatal Axotomy In Vivo

To confirm that the neurotrophic actions of CT-1 were not limited to cultured neurons, we tested its ability to protect neonatal rat motoneurons in vivo. Sciatic nerves of rat pups were sectioned on postnatal day 2, and a cupule containing a Fluorogold tracer plus the factor to be tested was applied directly to the nerve stump; 1, 2, and 3 weeks later, the rats were sacrificed, and the surviving fluorescently labeled motoneurons were counted on the operated side.

The initial number of sciatic motoneurons in the newborn rat was determined by applying Fluorogold alone to the cut sciatic nerve and sacrificing the rats 60–70 hr later; the value thus obtained was 1385 ± 150 (n = 4). A majority of these motoneurons had died by 1 week after axotomy: only 449 ± 74 (n = 5) motoneurons were maintained in vehicle-treated animals, indicating that approximately 936 cells had degenerated.

When animals were treated with CT-1 at 0.7 mg/ml, the number of motoneurons determined at 1 week (884 ± 47, n = 6) was significantly greater than in controls. Therefore, application of CT-1 can rescue about 46% of the cells that would normally have died during this period. The short-term rescue effects of CT-1 were very similar to those of LIF at this same concentration (883 ± 164, n = 3). Furthermore, the efficacy of CT-1 was not significantly changed when applied at 0.3, 0.7, or 1.5 mg/ml.

As with all other neurotrophic factors tested using this protocol (Vejsada et al., 1995), protection afforded by a single dose of CT-1 decreased with time (Figure 5). Nonetheless, a supramaximal dose of 1.5 mg/ml caused a significant rescue of motoneurons above the control levels at 1, 2, and 3 weeks. These long-term partial survival effects obtained with CT-1 have never been obs rved with CNTF or LIF (Vejsada et al., 1995).

Th CT-1 Rec ptor n Moton urons Has a GPI-Link d Comp n nt

The secr tion of CT-1 by mbryonic muscle, and its potent neurotrophic activity on motoneurons in vitro and

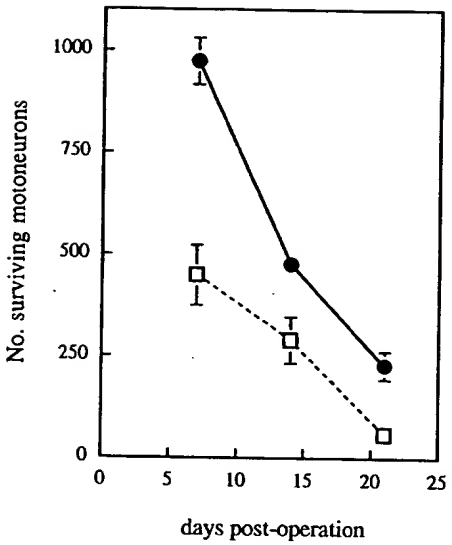


Figure 5. A Single Application of CT-1 Has Long-Term Protective Effects on Survival of Axotomized Neonatal Sciatic Motoneurons

The time course of motoneuron survival in the presence (closed circles) or absence (open squares) of 1.5 mg/ml CT-1. The neurotrophic factor (together with Fluorogold) was applied to the central stump of the cut sciatic nerve in 2-day-old rats. The number of motoneurons was determined 1, 2 or 3 weeks later. Motoneuron counts are expressed as mean ± SEM.

in vivo, raised the possibility that CT-1 might be a physiological survival factor for embryonic motoneurons. Two cytokine receptor subunits, LIFRβ and CNTFRα, were recently shown to be essential for the survival of motoneurons in knockout mice (DeChiara et al., 1995; Li et al., 1995b). We therefore designed experiments to test the hypothesis that CT-1 might be the physiological ligand for these receptors.

One characteristic property of the CNTFR complex is the presence of the GPI-linked CNTFRα subunit. As a first step toward testing the possibility that CT-1 might act through the CNTFR complex, we tested the capacity of phosphatidylinositol-specific phospholipase C (PIPLC) to inhibit the CT-1 survival response. PIPLC can specifically cleave the GPI linkage that attaches certain molecules to the surface of living cells (Low et al., 1986). Purified motoneurons in suspension were treated in complete culture medium for 2 hr at 37°C with 1–2 μg/ml PIPLC (or an equivalent volume of phosphate-buffered saline [PBS]) before being seeded in 16 mm wells in the presence or absence of different trophic factors. PIPLC was replaced in appropriate wells at 1–2 μg/ml at 0 hr and 14 hr of culture; motoneuron survival was quantified after 24–48 hr (Figures 6A and 6B). As expected, motoneuron survival in basal medium or in the presence of 1 ng/ml BDNF, which acts through the transmembrane TrkB receptor, did not differ significantly between PIPLC-treated and control motoneurons (in six independent experiments, the mean number of PIPLC-treated motoneurons that survived in 1 ng/ml BDNF was 107% ± 6% of the number of untreated motoneurons). PIPLC is not therefore toxic for motoneurons at this conc ntration (similar results were obtained using 5 μg/ml PIPLC; data not shown).

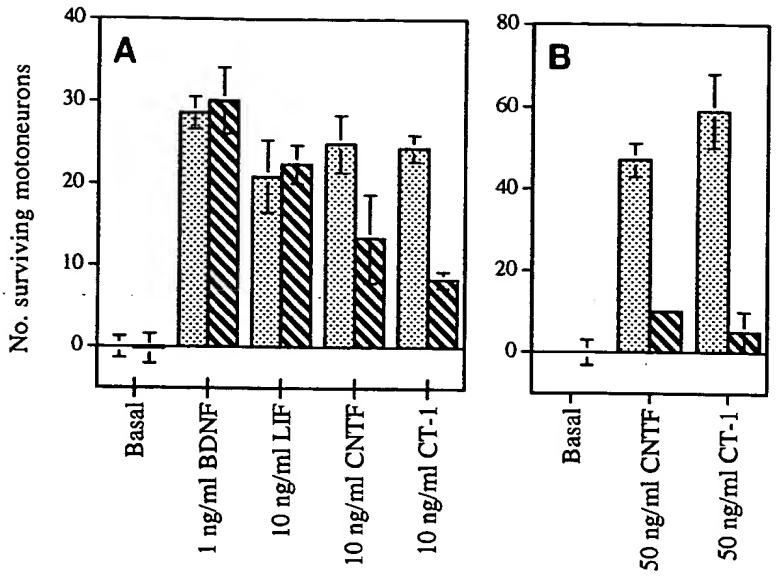


Figure 6. CT-1 Action on Motoneurons Requires a GPI-Linked Component

Purified motoneurons were preincubated with PIPLC (striped bars) or with PBS (stippled bars) and then seeded in the presence of the indicated concentrations of neurotrophic factors.

(A) PIPLC was used at 1 μg/ml and motoneuron survival was counted after 30 hr.

(B) In an independent experiment, PIPLC was used at 2 μg/ml and higher concentrations of neurotrophic factor were applied. To facilitate comparison, survival values were corrected for basal survival in Neurobasal medium in the corresponding experiment; they are expressed as the mean ± SEM of four counts in two independent wells.

Subsequently, the survival response in the presence of different cytokines was tested (Figure 6A). The number of motoneurons surviving in 10 ng/ml LIF remained unchanged after treatment with 1 μg/ml PIPLC, whereas the response to 10 ng/ml CNTF was decreased as expected. Strikingly, the response to 10 ng/ml CT-1 was reduc d to the same extent (Figure 6A). In subsequent experiments, PIPLC was used at 2 μg/ml: even greater reductions in CNTF- and CT-1-dependent survival were observed, even when cytokines were applied at 50 ng/ml (Figure 6B). On average (n = 3), the ratios of the numbers of surviving motoneurons in the presence and absence of 2 μg/ml PIPLC were 112% ± 23% (LIF), 20% ± 3% (CNTF), and 23% ± 8% (CT-1).

These data show that a GPI-linked component is required for CT-1 action in this system. This component may contribute to the CT-1 receptor complex in a manner analogous to that of the CNTFRα to the CNTFR complex. The incomplete loss of CT-1 sensitivity in PIPLC-treated motoneurons may reflect incomplete access of PIPLC to the GPI linkage, as suggested by the greater eff ct observed using 2 μg/ml PIPLC (Figures 6A and 6B), or action of CT-1 through the residual LIFR still present on these cells (Pennica et al., 1995b).

CT-1 Is N t a Ligand for the CNTFRα Subunit

Sinc CT-1 is related to CNTF, an obvious candidate for the putativ GPI-linked component revealed by PIPLC treatment was the CNTFRα subunit, the GPI-linked subunit that is necessary for signaling through the gp130/LIFR complex. We therefore directly examined, by several methods, the ability of CT-1 to bind this subunit.

A soluble form of the rat CNTFR (sCNTFR) was expressed as its extracellular domain containing a carboxy-t rminal histidine tag and purified by chelate chromatography (see Experimental Procedures). To confirm that th recombinant sCNTFRα was biologically active, we assessed its ability to cause hypertrophy of neonatal cardiac myocytes in the pres nce of its ligand. This hypertrophy assay was originally used to isolate CT-1 by expression cloning: both LIF and CT-1 induce myocyte hypertrophy, whereas CNTF has little or no activity (Pennica et al., 1995a). This difference in activity is presumably due to the lack of expression of CNTFRα on myocytes. When CNTF was added to cardiac myocytes alone, no hypertrophy was observed. However, when the same concentration of CNTF was added in the presence of 25 nM or 50 nM sCNTFR, then hypertrophy was close to maximal (scores 6.0 and 6.5, respectively) and similar to that reported for LIF and CT-1 (Figure 7A). Thus, the purified sCNTFR was biologically active and allowed CNTF to signal through the LIFR complex as previously described (Davis et al., 1993).

We first tested the ability of CT-1 to bind directly to sCNTFRα. ^{125}I-labeled CT-1 (0.7 nM) was incubated with sCNTFRα (40 nM) for 2 hr at room temperature, and any complexes formed were captured using nickel agarose beads. No specific binding could be detected (data not shown). Using an identical protocol, strong specific binding of labeled CT-1 to sLIFR, and of labeled CNTF to sCNTFRα, were observed as expected, demonstrating that all components were functional (data not shown). To exclude the possibility that CT-1 bound to sCNTFRα might be lost during precipitation of the complex, we performed incubations of labeled CT-1 with sCNTFRα or sLIFR in the same conditions, except that after 2 hr the cross-linker 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide/N-hydroxysulfosuccinimide (EDC/sulfo-NHS) was added for 30 min. After analysis by SDS–PAGE, no cross-linking of CT-1 to sCNTFRα could be detected, whereas CT-1 and sLIFR gave a radioactive cross-linked product of the appropriate size that disappeared when the incubation was performed in the presence of 1 μM unlabeled CT-1, but remained in the presence of 1 μM unlabeled CNTF (data not shown).

Since we could detect no direct binding of CT-1 to sCNTFRα, we used functional assays to detect possible interactions between them. First, the hypertrophy of cardiac myocytes was assayed in the presenc of differ nt CT-1 concentrations ranging from 1 pM (no hypertrophy) to 200 pM (near-maximal hypertrophy). No chang in

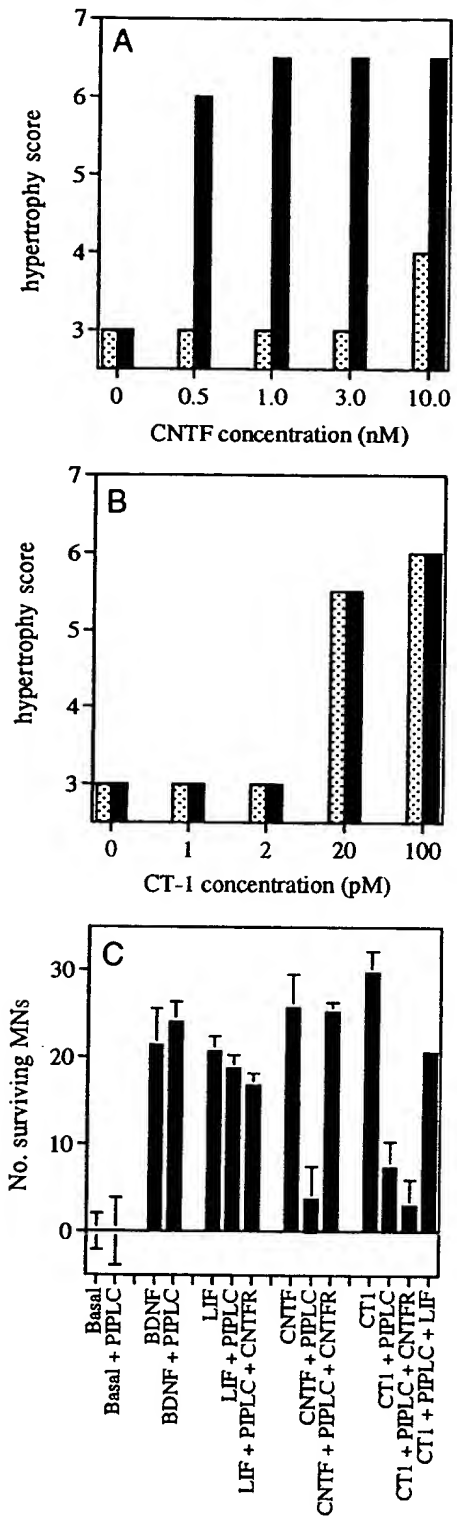


Figure 7. Soluble CNTFRα Can Mediate the Actions of CNTF on Myocytes and Motoneurons, but Does Not Potentiate CT-1 Action

(A and B) Hypertrophic effects on neonatal rat ventricular myocytes of CNTF (A) or CT-1 (B) tested alone at indicated concentrations (stippled bars) or in the presence of 25 nM sCNTFRα (closed bars). A score of 3 is no hypertrophy; 7 is maximal hypertrophy. Note that the dose-response for CT-1 is not displaced by sCNTFRα.

(C) Motoneuron survival values (corrected for those in basal medium) after 52 hr of culture in the presence of indicated substances. Neurotrophic factors were used at: 1 ng/ml (BDNF), 50 ng/ml (LIF, CNTF, and CT-1). PIPLC was used at 2 μg/ml as described in the text. Soluble CNTFRα (25 nM) reconstituted the effect of CNTF, but not of CT-1. Values are mean ± SEM of four counts in two independent wells.

any of the hypertrophy scores was observed when the same CT-1 concentrations were tested in the presence of biologically active concentrations of sCNTFRα (Figure 7B). Thus, the hypertrophy response of the myocytes to CT-1 is not potentiated by sCNTFRα.

A further demonstration of the inability of sCNTFRα to facilitate CT-1 action was provided by analysis of purified motoneurons treated with PIPLC. As described above, PIPLC-treated motoneurons responded only poorly to 50 ng/ml CNTF (Figure 7C). However, in the presence of 25 nM sCNTFRα, the CNTF response of PIPLC-treated motoneurons was completely reconstituted. In contrast, sCNTFRα caused no increase in the response of PIPLC-treated motoneurons to CT-1, although motoneurons treated with PIPLC and CT-1 were still capable of a normal response to LIF (Figure 7C). sCNTFRα itself had no effect on the response to LIF (Figure 7C). Therefore, even in the specific environment of the motoneuron membrane, sCNTFRα cannot mediate interactions of CT-1 with the LIFR complex.

It remained possible that CT-1 exerted its biological effects through CNTFRα, but was unable to interact functionally with the soluble form of the receptor. We therefore tested the ability of CT-1 to compete for the binding of radiolabeled CNTF to the rat adrenal pheochromocytoma PC12 cell line (Figure 8A). These cells have been reported to bind CNTF and form a binding complex composed of ligand, CNTFRα and gp130 (Wong et al., 1995). Excess unlabeled CNTF reduced binding of radiolabeled CNTF on PC12 cells to background levels. In contrast, CT-1 and LIF were unable to compete for CNTF binding even at a 1000-fold molar excess (Figure 8A). Similar results were obtained using human 293 cells (Figure 8B). Furthermore, binding of radiolabeled CT-1 to M1 cells was reduced to background levels by incubation with unlabeled CT-1 or LIF, but was only partially affected by incubation with unlabeled CNTF (Figure 8C). Whereas treatment of PC12 and 293 cells with PIPLC significantly reduced CNTF binding (Figures 8A and 8B), binding of CT-1 to M1 cells was not affected by PIPLC (Figure 8C). In agreement with this, PIPLC treatment did not affect the hypertrophic activity of CT-1 for cardiac myocytes (data not shown).

Taken together, these data suggest that CT-1 is not a ligand for the CNTFRα subunit. The GPI-linked component of the CT-1 receptor complex on motoneurons may therefore be a novel cytokine receptor subunit.

Discussion

Our results make cardiotrophin-1 a strong candidate as a physiological motoneuron survival factor. It supports

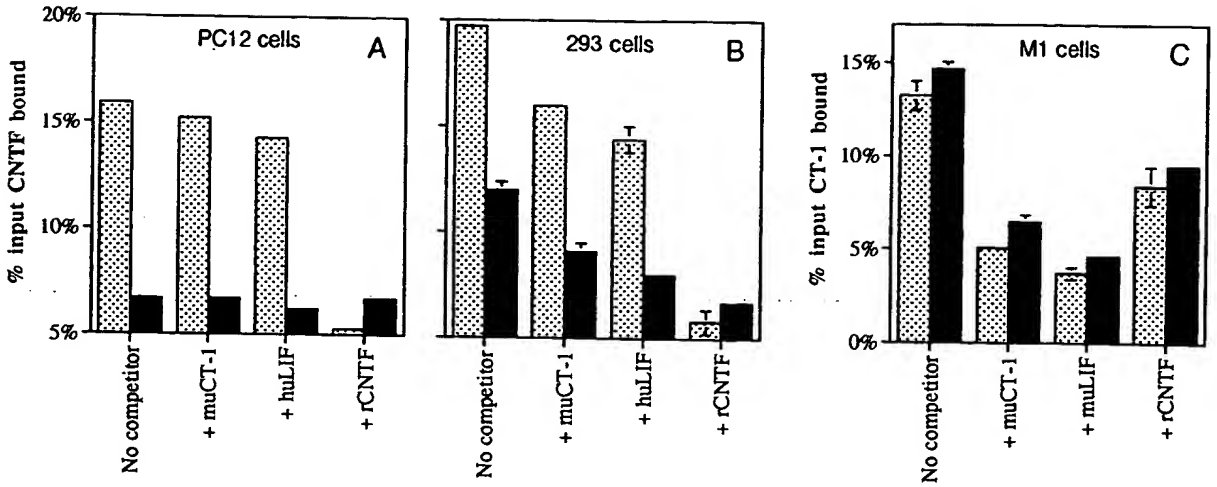


Figure 8. Competition between Cytokines for Their Binding Sites

Rat PC12 cells (A), human kidney 293 cells (B), and mouse M1 cells (C). [^{125}I]rCNTF (A and B) and [^{125}I]mCT-1 (C) were incubated in duplicate with PC-12 cells (4×10^5 cells per reaction), 293 cells (5×10^6 cells), and M1 (10×10^6 cells) in the presence of a 1000-fold molar excess of the indicated competitors as described in Experimental Procedures. The same experiments were performed in each case with untreated cells (stippled bars) and cells preincubated with 2 μg/ml PIPLC (closed bars). Results are the mean ± range of two reactions.

the survival and maturation of a discrete fraction of motoneurons on a long-term basis, is synthesized and secreted by embryonic muscle, and can keep lesioned motoneurons alive in vivo. Furthermore, it binds to the LIFRβ subunit, which is expressed by embryonic motoneurons and is known to be necessary for the survival of a significant fraction of them.

CNTFR and LIFR knockout mice show a similar extent of motoneuron loss at birth (33% and 43% in lumbar spinal cord, 41% and 37% in facial nucleus, respectively) (DeChiara et al., 1995; Li et al., 1995b). It is indeed possible that the same motoneurons are lost in each mutant. Although prudence is required in comparing in vivo and in vitro data, it is striking that the percentages of motoneurons that survive in the presence of CT-1 in our long-term cultures (43%) and in vivo after axotomy (46%) are very similar to the fraction lost in CNTFR and LIFR knockout mice. Moreover, in short-term cultures of motoneurons, CNTF has been reported to support approximately half of the total population (Arakawa et al., 1990; Martinou et al., 1992; Henderson et al., 1994). In all these experimental systems, motoneuron death is continuing (at least at a reduced rate) at the time at which survival is estimated. It would therefore be inappropriate to attribute too great a significance to the absolute value of 45%. N vertheless, the data are consistent with the idea that a discr te subpopulation of motoneurons is capable of responding to cytokines and requires them for survival. Most motoneurons in this population must express both CNTFRα and LIFRβ subunits since, using E14 rat motoneurons, optimal concentrations of CNTF gave similar long-term survival values to LIF, and combinations of different cytokines did not further increase survival. It is not known whether only a fraction of moton urons in situ xpr ss different components of the cytokine rec ptor complex. It will thus be important to determine wh ther cytokine-responsive motoneurons differ from other motoneurons in this respect, or in terms of other markers involv d in signaling and survival.

Could CT-1 be the physiological ligand for the CNTFR and LIFR, which are known to be required for survival of some embryonic motoneurons? Our data clearly demonstrate that a component of the receptor or signaling system for CT-1 at the surface of cultured motoneurons is sensitive to PIPLC, under conditions in which this enzyme shows no nonspecific toxicity (Figure 6). Since the response to CNTF, but not to LIF, is also affected by PIPLC, it is probable that the CT-1 receptor, like the CNTFR, has an essential GPI-linked component. Using a variety of approaches, we were unable to detect binding of CT-1 to the CNTFRα subunit (Figures 7 and 8). Although we cannot completely rule out the possibility that CT-1 can act through CNTFRα in the specific environment of the motoneuron membrane, the simplest hypothesis would be that CT-1 action requires a novel GPI-linked α subunit. Our tentative conclusion, therefore, is that the cytokine whose existence is implied by motoneuron loss in the CNTFR knockout mice is not CT-1. Interestingly, binding of CT-1 to non-neuronal cells expressing LIFR is not PIPLC-sensitive, and PIPLC treatment did not reduce the ability of CT-1 to induce cardiac myocyte hypertrophy. These data suggest that the novel GPI-linked component may not be present on all CT-1-responsive cell types. Further analysis of the physiological role of CT-1 in the spinal cord will involve identification of the GPI-linked component, as well as inactivation of the CT-1 gene itself.

Consistent with a role in regulating motoneuron numbers during development, levels of CT-1 mRNA are high in limb bud at the beginning of cell death and subsequently fall off steeply (Figure 1). Similar developmental regulation was reported for levels of LIF mRNA, which fall rapidly between E17 and birth in mouse hind leg muscles (Kwon et al., 1995). Using an antiserum to CT-1, Sheng t al. (1996) recently described high lev ls f CT-1 protein at E15.5 in sk letal muscle, the target tissue of motoneurons, but did n t compare levels at later stages. The presence f CT-1 mRNA in adult skeletal muscl

has been demonstrated by Northern blots in both mouse (Pennica et al., 1995a) and human (Pennica et al., 1996). We used immortalized cell lines to demonstrate that newly formed myotubes synthesize high levels of CT-1 mRNA. GDNF, another factor thought to play a role in motoneuron development is predominantly expressed in Schwann cells at E14.5 (although GDNF mRNA appears in muscle at later stages) (Henderson et al., 1994; Trupp t al., 1995). It is possible that CT-1 and GDNF may interact to promote the survival of motoneurons in vivo; th potential mechanisms of this interaction are currently under study.

The absence of a conventional signal peptide in the CNTF molecule initially led to the hypothesis that it may serve as a "lesion" factor, to be released upon injury (S ndtner et al., 1990). Although evidence for this hypothesis has not been forthcoming, it was important to demonstrate that CT-1, which also lacks a signal peptide, can be secreted by muscle cells and thereby become potentially available to the motoneuron. Using an affinity-purified polyclonal antibody to CT-1, we detected a 30 kDa species corresponding to mature CT-1 in the supernatant of healthy cultures of C2/C7 myotubes. No dying cells were microscopically apparent in these cultures, and thus the CT-1 is unlikely to have been released by cell lysis. Furthermore, the estimated levels of CT-1 (1–5 ng/ml) are consistent with the idea that it may be a major contributor to the trophic activity of myotube-conditioned media. This provides direct evidence for secretion of a potential neurotrophic factor for motoneurons by their target tissue, skeletal muscle.

We used an experimental model of motoneuron degeneration in vivo to show that, like other cytokines of this family, CT-1 can significantly reduce motoneuron death brought about by neonatal axotomy. These results make CT-1 an important addition to the list of neurotrophic factors that may potentially be of use in slowing pathologic motoneuron loss in humans, but several points remain to be addressed. First, since CT-1 is a cytokine with actions in other tissues such as heart (Pennica et al., 1995a) and liver (Peters et al., 1995), it will be necessary to limit potential side effects by localized delivery. Second, the "rescue" effect described here decreases after the first week following axotomy. It is likely that, as for other neurotrophic factors tested using this model (Vejsada et al., 1995; R. V. and A. K., unpublished data), more durable effects will be obtained by the use of combinations of neurotrophic factors and of improved methods of delivery.

In conclusion, cardiotrophin-1, originally identified for its ffects on hypertrophy of cardiac myocytes, has effects on motoneurons in vitro and in vivo that are of potential importance in understanding the normal development f the motor unit and in attempting to reduce motoneuron loss in human neurodegenerative disease.

Experimental Procedures

In Situ Hybridization

Mouse E13.5 embryos were immersion-fixed overnight at 4°C in 4% paraformaldehyde and then cryoprotected overnight in 15% sucrose. Sections (16 μm) were processed for in situ hybridization for CT-1 by a modification of the method previously described (Phillips et al., 1990). [^{33}P]UTP-labeled RNA probes were generated as described (Melton et al., 1984). Simultaneous hybridization with two probes to different regions of CT-1 mRNA was performed. The pairs of sense and antisense probes were synthesized from cloned 161 bp and 188 bp cDNA fragments (nucleotides 20–180 and 645–832, respectively; Pennica et al., 1995a) using T7 polymerase.

cDNA Preparation

Total RNA was isolated using the Trizol reagent (GIBCO-BRL). A sample of each RNA was treated for 2 hr with RNase-free DNase I (Boehringer Mannheim) and then extracted with Trizol and precipitated with isopropanol. Pellets were taken up in 48 μl of reverse transcriptase mix containing the following: 200 μM dNTP mix (Pharmacia), 10 mM DTT, 0.5 μg of oligo(dT) (GIBCO-BRL), 1× reaction buffer (GIBCO-BRL). Reactions were incubated for 10 min at 70°C, chilled on ice, and divided into two aliquots, one of which received 400 U of Superscript II (GIBCO-BRL), the other serving as the RT-negative control. Both aliquots were incubated at 42°C for 1 hr.

PCR Reactions

To normalize the levels of each cDNA, control GAPDH PCR reactions were carried out for 27 cycles as described (Henderson et al., 1994). PCR reactions for CT-1 cDNA (34 cycles) were then performed in a final volume of 50 μl containing the following: 1 μg of each primer, 200 μM dNTP mix, 60 mM Tris–HCl (pH 9.5), 15 mM $(NH_4)_2SO_4$, 1.5 mM $MgCl_2$, 0.05% W-1 (GIBCO-BRL), and 1 U of Taq DNA polymerase (GIBCO-BRL). The following specific primers were used: forward primer CGGCCAACAGCACTGCAGGCATC (nucleotides 504–526 of the mouse CT-1 cDNA sequence; GenBank accession number U18366); reverse primer AAGCTCCCTGCAGAGAGGAGAGC (nucleotides 785–763); and internal probe for Southern blots GTCGCCCTC TGTGCGGCTCACCCACTC (nucleotides 598–572). PCR products were electrophoresed on a 6% acrylamide gel, and the results were analyzed by EtBr staining. After electroblotting, all amplified fragments hybridized strongly to the internal probe (data not shown).

Detection of CT-1 on Western Blots

Cell culture supernatants were concentrated by centrifugation through Centricon membranes and loaded onto 10% polyacrylamide gels. Pure recombinant CT-1 was used as a marker together with Amersham Rainbow markers. After electrophoresis, gels were blotted onto nitrocellulose membranes and stained with Ponceau Red. Since the primary antibody showed some reaction with the high levels of serum albumin present in the conditioned media, membranes were cut just below the albumin band at 66 kDa, and only the lower part processed for CT-1 immunoreactivity. Prior to addition of antibody, membranes were prehybridized in 1% nonfat milk, 5% goat serum for 30 min. Affinity-purified anti-CT-1 antibodies (3 μg/ml) were added in the prehybridization solution and incubated at 25°C for 1 hr. The membranes were washed three times with PBS containing 0.5% Tween 20 before addition of a 1:2000 dilution of a peroxidase-linked donkey anti-rabbit antibody. Following incubation in the second antibody for 1 hr the filters were washed an additional three times with PBS containing Tween 20 before detection of the antigen using standard ECL (Boehringer).

Preparation of Affinity-Purified CT-1 Antibody

We mixed 4.4 mg of dialyzed and concentrated CT-1 with controlled pore glass (CPG) glyceryl affinity resin (Sigma) for 24 hr at 4°C in the presence of $NaBH_3CN$ (to stabilize the binding of CT-1 to the resin). The resin was washed several times with 0.05 M sodium carbonate (pH 8.5) and mixed with $NaBH_3CN$ and 1 M ethanolamine (to block unbound sites) for 24 hr at 4°C. This treated resin was then packed into an FPLC column. The column was preeluted with 0.1 M Na-acetate (pH 2.8), followed by equilibration with load buffer (PBS, 0.5 M NaCl). Rabbit antiserum to CT-1 was sterile filtered and applied to the column at a flow rate of 2.0 ml/min. The nonspecific proteins were washed off the column with load buffer, and the CT-1-specific antibodies were eluted as 0.5 ml fractions with 0.1 M Na-acetate (pH 2.8). The final antibody preparation was either sterile filtered or stored with 0.01% thimerosal.

Motoneuron Purification and Culture

Spinal motoneurons were purified from ventral spinal cords of E14 rat embryos essentially as described (Henderson et al., 1995). In brief, cells were dissociated after trypsin treatment and centrifuged over a 6.5% metrizamide cushion. The large cells remaining above the cushion were further fractionated by immunopanning on dishes coated with the 192 antibody (Chandler et al., 1984), which recognizes the low affinity NGF receptor and is specific to motoneurons at this age (Yan and Johnson, 1988). Contaminating cells were removed by washing and specifically bound motoneurons eluted using an excess of 192 antibody. Purified motoneurons were collected by centrifugation through a bovine serum albumin (BSA) cushion. Typical yields were 10,000 large motoneurons per E14 spinal cord. No exact figures are available for the total number of motoneurons in embryonic rodent spinal cord, but one lumbar motor column in neonatal mice contains on the order of 3000 motoneurons (DeChiara et al., 1995; Li et al., 1995b), suggesting that the total number may not be far in excess of 25,000.

Purified motoneurons were seeded in dishes coated with polyornithine–laminin (1500–2000 per 35 mm dish; 1000 per 16 mm well). Culture medium ("basal medium") was Neurobasal (GIBCO-BRL), supplemented with the B27 supplement (GIBCO-BRL), horse serum (2% v/v), L-glutamine (0.5 mM), and 2-mercaptoethanol (25 μM). L-glutamate (25 μM) was added to the medium for the first 4 days of culture and subsequently omitted. Subsequently, every 4–5 days, 1.5 ml of medium was removed and replaced with 1.5 ml fresh medium containing sufficient quantities of the indicated neurotrophic factor to supplement the 2 ml of medium in the dish. For longer term cultures, precautions were taken to maintain high humidity, so as to prevent concentration of medium components by evaporation.

Quantification of Motoneuron Survival in Culture

Motoneuron survival was quantified by direct counting under phase-contrast in a predetermined area of 1.5 mm^2 in the center of the dish. All motoneurons that appeared alive by phase-contrast also were labeled by the vital dye AM-calcein (data not shown). Initial attachment of motoneurons was identical in all conditions. To compare data from different experiments in a quantitative manner, the number of motoneurons that developed initially in the presence of 100 pg/ml GDNF was determined by counting neurons with neurites >4 cell diameters in length after 16 hr of culture. Unless otherwise indicated, this figure was taken as 100% survival. At later stages, only large Islet-positive neurons with long axon-like processes (>80% of cells present) were counted. The small motoneurons we have described (Henderson et al., 1993b) were rare or absent in these conditions and in general did not survive in long-term cultures.

Immunofluorescence Labeling of Cultured Motoneurons

Motoneurons were fixed using 4% paraformaldehyde, 0.1% glutaraldehyde in PBS at 4°C for 30 min and then washed. After quenching of excess aldehyde groups with 50 mM lysine, cells were permeabilized using 0.1% Triton X-100, and nonspecific binding sites were blocked by incubation for 2 hr with PBS containing horse serum (10% v/v) and BSA (2% w/v). Subsequently, first antibody was added at the indicated dilution and allowed to bind overnight at 4°C. After washing, dishes were treated with appropriate secondary antibodies (dilution 1:100) coupled to the indicated fluorophores. Primary antibodies were as follows: mouse monoclonal anti-Islet 2D6 (Developmental Hybridoma Bank, dilution 1:2; data not shown) and affinity-purified rabbit polyclonal antibodies to the 200 kDa neurofilament subunit (Sigma, dilution 1:500).

Sciatic Nerve Axotomy in Newborn Rats

In anesthetized 2-day-old Sprague–Dawley rats, the sciatic nerve was transected on one side and a small cupule containing approximately 2.5 μl of neurotrophic factor or 0.7 mg/ml BSA was applied to the proximal nerve stump. To specifically label the sciatic motoneuron pool, the fluorescent retrograde tracer Fluorogold (2.5%; Fluorochrome, Englewood, Colorado) was simultaneously added into the tube. The incision was sutured and the rats returned to their mother.

Histology and Motoneuron Counting

At the indicated survival times, rats were sacrificed by an overdose of phenobarbital. Following transcardiac perfusion with 4% paraformaldehyde in PBS, the lumbosacral spinal cord was removed, cryoprotected in sucrose-PBS solutions, and sectioned (30 μm) on a cryostat. Serial sections were cover-slipped with Eukitt and viewed under a Reichert–Jung fluorescent microscope with a UV filter at 125× to 200× magnification. Fluorogold-labeled sciatic motoneurons were localized in the ventral horn of spinal segments from caudal L3 through to cranial L6. Profiles of labeled cells were counted on each section (i.e., 90–120 sections per cord, depending on the age); no correction factor was applied to the counts. The values shown are means ± SEMs from at least three animals.

Preparation of Soluble CNTFR

The cDNA coding for the CNTFRα subunit was generated from E14 mouse brain by RT–PCR. PCR primers were designed to truncate the amino acid sequence before the GPI linkage site (amino acids 1–335) and a C-terminal tag encoding six histidine residues was added. Sequencing of the cDNA in the mammalian expression vector pRK5 (Suva et al., 1987) confirmed the sequence was correct. The expression plasmid was transfected into 293 cells and after 4 days the conditioned medium was concentrated 20-fold (Centriprep 10, Amicon). The tagged protein was isolated by Ni^{2+}–nitrilo–triacetic acid–agarose (QIAGEN) as described (Pennica et al., 1995a).

Myocyte Hypertrophy Assay

Substances were tested as described previously (Pennica et al., 1995a). In brief, hearts were excised from 1-day-old Sprague–Dawley rats. Ventricular cardiac myocytes were isolated by collagenase digestion and Percoll gradient purification. The myocytes were diluted into serum-free DMEM/F12 supplemented with 10 μg/ml transferrin, 1 μg/ml insulin, 1 μg/ml aprotinin, 2 mM glutamine, 100 U/ml penicillin G, and 100 μg/ml streptomycin and plated at a final density of 1.5×10^4 cells per well of a 96-well flat-bottomed plate previously coated with 10% fetal calf serum in DMEM/F12. After 24 hr at 37°C, test substances were added. After 2 days, the cells were fixed and stained with 0.5% crystal violet. The hypertrophy score was assessed on a scale of 1 to 7 by microscopic evaluation. The increase in myocyte cell size, expressed as hypertrophy score, is dose-dependent and is accompanied by an increase in atrial natriuretic peptide production. Untreated cells are scored as 3. Toxic effects are scored from 0–2. A score of 7 indicates maximal hypertrophy such as that induced by 0.1 mM phenylephrine.

Binding Assays

Mouse CT-1 and rat CNTF (R&D Systems) were iodinated as described (Pennica et al., 1995b) to specific activities of 1000 Ci/mmol and 750 Ci/mmol, respectively, using Enzymobeads (Bio-Rad). For binding assays, 0.14 nM ^{125}I-labeled rat CNTF and 0.09 nM ^{125}I-labeled CT-1 were used. Cells were incubated in a 150 μl volume with gentle agitation in the presence of iodinated ligand in the presence or absence of 100 nM of unlabeled competitor for 2 hr at room temperature. Unbound counts were removed from the cell pellets by two washes with ice-cold PBS with 0.1% BSA and the pellets counted in a gamma counter (Isodata)

Acknowledgments

We thank O. deLapeyrière and other members of INSERM Unité 382 for many helpful discussions. We are grateful to A. Nuijens and G. Bennett for preparation of the affinity-purified CT-1 antibodies, D. Shelton and W.-P. Chan for rat CNTF, M. Rodriguez for PIPLC, C. Pinset, M. Buckingham, D. Paulin, and M. Pinçon-Raymond for gifts of muscle cell lines, G. Rougon for the C6 glioma, N. F. Paoni for help with the CT-1 protein purification, P. Weber for help with photography, and D. I. Wood for help with the graphics. This work was funded by grants to C. E. H. from INSERM, the Association Française contre les Myopathies, the Institut pour la Recherche sur la Moelle Epinière, and to A. K. from the Swiss National Science Foundation, AFM, Sandoz Research Foundation, and Roche Research Foundation. Research fellowships were provided by the Wellcome Trust (R. A. P.) and the European Molecular Biology Organization (K. D.).

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 USC Section 1734 solely to indicate this fact.

Received May 23, 1996; revised June 18, 1996.

References

Arakawa, Y., Sendtner, M., and Thoenen, H. (1990). Survival effect of ciliary neurotrophic factor (CNTF) on chick embryonic motoneurons in culture: comparison with other neurotrophic factors and cytokines. J. Neurosci. *10*, 3507–3515.

Catala, F., Wanner, R., Barton, P., Cohen, A., Wright, W., and Buckingham, M. (1995). A skeletal muscle–specific enhancer regulated by factors binding to E and CArG boxes is present in the promoter of the mouse myosin light-chain 1A gene. Mol. Cell. Biol. *15*, 4585–4596.

Chandler, C.E., Parsons, L.M., Hosang, M., and Shooter, E.M. (1984). A monoclonal antibody modulates the interaction of nerve growth factor with PC12 cells. J. Biol. Chem. *259*, 6882–6889.

Davis, S., Aldrich, T.H., Stahl, N., Pan, L., Taga, T., Kishimoto, T., Ip, N.Y., and Yancopoulos, G.D. (1993). LIFRβ and gp130 as heterodimerizing signal transducers of the tripartite CNTF receptor. Science *260*, 1805–1808.

DeChiara, T.M., Vejsada, R., Poueymirou, W.T., Acheson, A., Suri, C., Conover, J.C., Friedman, B., McClain, J., Pan, L., Stahl, N., Ip, N.Y., Kato, A., and Yancopoulos, G.D. (1995). Mice lacking the CNTF receptor, unlike mice lacking CNTF, exhibit profound motor neuron deficits at birth. Cell *83*, 313–322.

Escary, J.L., Perreau, J., Dumenil, D., Ezine, S., and Brûlet, P. (1993). Leukaemia inhibitory factor is necessary for maintenance of haematopoietic stem cells and thymocyte stimulation. Nature *363*, 361–364.

Hamburger, V. (1992). History of the discovery of neuronal death in embryos. J. Neurobiol. *23*, 1116–1123.

Henderson, C.E. (1995). Neurotrophic factors as therapeutic agents in ALS. Adv. Neurol. *68*, 235–240.

Henderson, C.E. (1996). Role of neurotrophic factors in neuronal development. Curr. Opin. Neurobiol. *6*, 64–70.

Henderson, C.E., Bloch-Gallego, E., Camu, W., Gouin, A., and Mettling, C. (1993a). Neurotrophic factors in development and plasticity of spinal neurons. Restor. Neurol. Neurosci. *5*, 15–28.

Henderson, C.E., Camu, W., Mettling, C., Gouin, A., Poulsen, K., Karlaloo, M., Rullamas, J., Evans, T., McMahon, S.B., Armanini, M.P., Berkemeier, L., Phillips, H.S., and Rosenthal, A. (1993b). Neurotrophins promote motor neuron survival and are present in embryonic limb bud. Nature *363*, 266–270.

Henderson, C.E., Phillips, H.S., Pollock, R.A., Davies, A.M., Lemeulle, C., Armanini, M.P., Simpson, L.C., Moffet, B., Vandlen, R.A., Koliatsos, V.E., and Rosenthal, A. (1994). GDNF: a potent survival factor for motoneurons present in peripheral nerve and muscle. Science *266*, 1062–1064.

Henderson, C.E., Bloch-Gallego, E., and Camu, W. (1995). Purification and culture of embryonic spinal motoneurons. In Nerve Cell Culture: A Practical Approach, J. Cohen and G. Wilkin, eds. (London: Oxford University Press), pp. 69–81.

Ip, N.Y., McClain, J., Barrezueta, N.X., Aldrich, T.H., Pan, L., Li, Y., Wiegand, S.J., Friedman, B., Davis, S., and Yancopoulos, G.D. (1993). The α component of the CNTF receptor is required for signaling and defines potential CNTF targets in the adult and during development. Neuron *10*, 89–102.

Kwon, Y.W., Abbondanzo, S.J., Stewart, C.L., and Gurney, M.E. (1995). Leukemia inhibitory factor influences the timing of programmed synapses withdrawal from neonatal muscles. J. Neurobiol. *28*, 35–50.

Li, L., Wu, W., Lin, L.-F.H., Lei, M., Oppenheim, R.W., and Houenou, L.J. (1995a). Rescue of adult mouse motoneurons from injury-induced cell death by glial cell line-derived neurotrophic factor. Proc. Natl. Acad. Sci. USA *92*, 9771–9775.

Li, M., Sendtner, M., and Smith, A. (1995b). Essential function of LIF receptor in motor neurons. Nature *378*, 724–727.

Low, M., Ferguson, M.A.J., Futerman, A.H., and Silman, I. (1986). Covalently attached phosphatidylinositol as a hydrophobic anchor for membrane proteins. Trends Biochem. Sci. *11*, 212–215.

Martinou, J.-C., Martinou, I., and Kato, A. (1992). Cholinergic differentiation factor (CDF/LIF) promotes survival of isolated rat embryonic motoneurons in vitro. Neuron *8*, 737–744.

Masu, Y., Wolf, E., Holtmann, B., Sendtner, M., Brem, G., and Thoenen, H. (1993). Disruption of the CNTF gene results in motor neuron degeneration. Nature *365*, 27–32.

Melton, D.A., Krieg, P.A., Rebagliati, M.R., Maniatis, T., Zinn, K., and Green, M.R. (1984). Efficient *in vitro* synthesis of biologically active RNA and RNA hybridization probes from plasmids containing a bacteriophage SP6 promoter. Nucl. Acids Res. *12*, 7035–7056.

Oppenheim, R.W. (1991). Cell death during development of the nervous system. Annu. Rev. Neurosci. *14*, 453–501.

Oppenheim, R.W., Prevette, D., Yin, Q.W., Collins, F., and MacDonald, J. (1991). Control of embryonic motoneuron survival *in vivo* by ciliary neurotrophic factor. Science *251*, 1616–1618.

Oppenheim, R.W., Yin, Q.W., Prevette, D., and Yan, Q. (1992). Brain-derived neurotrophic factor rescues developing avian motoneurons from cell death. Nature *360*, 755–757.

Oppenheim, R.W., Houenou, L.J., Johnson, J.E., Lin, L.-F. H., Li, L., Lo, A.C., Newsome, A.L., Prevette, D.M., and Wang, S. (1995). Developing motor neurons rescued from programmed and axotomy-induced cell death by GDNF. Nature *373*, 344–346.

Pennica, D., King, K.L., Shaw, K.J., Luis, E., Rullamas, J., Luoh, S. M., Darbonne, W.C., Knutzon, D.S., Yen, R., Chien, K.R., Baker, J.B., and Wood, W.I. (1995a). Expression cloning of cardiotrophin-1, a cytokine that induces cardiac myocyte hypertrophy. Proc. Natl. Acad. Sci. USA *92*, 1142–1146.

Pennica, D., Shaw, K.J., Swanson, T.A., Moore, M.W., Shelton, D.L., Zioncheck, K.A., Rosenthal, A., Taga, T., Paoni, N.F., and Wood, W.I. (1995b). Cardiotrophin-1. Biological activities and binding to the leukemia inhibitory factor receptor/gp130 signaling complex. J. Biol. Chem. *270*, 10915–10922.

Pennica, D., Swanson, T.A., Shaw, K.J., Kuang, W.-J., Gray, C., Beatty, B.G., and Wood, W.I. (1996). Human cardiotrophin-1 protein and gene structure, biological and binding activities, and chromosomal localization. Cytokine *8*, 183–189.

Peters, M., Roeb, E., Pennica, D., Meyer zum Buschenfelde, K.H., and Rose, J.S. (1995). A new hepatocyte stimulating factor: cardiotrophin-1 (CT-1). FEBS Lett. *372*, 177–180.

Phillips, H.S., Hains, J.M., Laramee, G.R., Rosenthal, A., and Winslow, J.W. (1990). Widespread expression of BDNF but not NT3 by target areas of basal forebrain cholinergic neurons. Science *250*, 290–294.

Pinçon-Raymond, M., Vicart, P., Bois, P., Chassande, O., Romey, G., Varadi, G., Li, Z.L., Lazdunski, M., Rieger, F., and Paulin, D. (1991). Conditional immortalization of normal and dysgenic mouse muscle cells by the SV40 large T antigen under the vimentin promoter control. Dev. Biol. *148*, 517–528.

Pinset, C., Mulle, C., Benoît, P., Changeux, J.P., Chelly, J., Gros, F., and Montarras, D. (1991). Functional adult acetylcholine receptor develops independently of motor innervation in Sol8 mouse muscle cell line. EMBO J. *10*, 2411–2418.

Sagot, Y., Tan, S.A., Baetge, E., Schmalbruch, H., Kato, A.C., and Aebischer, P. (1995). Polymer encapsulated cell lines genetically engineered to release ciliary neurotrophic factor can slow down progressive motor neuronopathy in the mouse. Eur. J. Neurosci. *7*, 1313–1322.

Sendtner, M., Kreutzberg, G.W., and Thoenen, H. (1990). Ciliary neurotrophic factor prevents the degeneration of motor neurons after axotomy. Nature *345*, 440–441.

Sendtner, M., Schmalbruch, H., Stöckli, K.A., Carroll, P., Kreutzberg, G.W., and Thoenen, H. (1992a). Ciliary neurotrophic factor prevents degeneration of motor neurons in mouse mutant progressive motor neuronopathy. Nature *358*, 502–504.

Sendtner, M., Holtmann, B., Kolbeck, R., Thoenen, H., and Barde, Y.-A. (1992b). Brain-derived neurotrophic factor prevents the death of motoneurons in newborn rats after nerve section. Nature *360*, 757–759.

Sendtner, M., Götz, R., Holtmann, B., Escary, J.-L., Masu, Y., Carroll, P., Wolf, E., Brem, G., Brûlet, P., and Thoenen, H. (1996). Cryptic physiological trophic support of motoneurons by LIF revealed by double gene targeting of CNTF and LIF. Curr. Biol. *6*, 686–694.

Sheng, Z., Pennica, D., Wood, W.I., and Chien, K.R. (1996). Cardiotrophin-1 displays early expression in the murine heart tube and promotes cardiac myocyte survival. Development *122*, 419–428.

Stewart, C.L., Kaspar, P., Brunet, L.J., Bhatt, H., Gadi, I., Kontgen, F., and Abbondanzo, S.J. (1992). Blastocyst implantation depends on maternal expression of leukaemia inhibitory factor. Nature *359*, 76–79.

Stöckli, K.A., Lillien, L.E., Naher, N.M., Breitfeld, G., Hughes, R. A., Raff, M.C., Thoenen, H., and Sendtner, M. (1991). Regional distribution, developmental changes, and cellular localization of CNTF-mRNA and protein in the rat brain. J. Cell Biol. *115*, 447–459.

Suva, L.J., Winslow, G.A., Wettenhall, R.E., Hammonds, R.G., Moseley, J.M., Diefenbach, J.H., Rodda, C.P., Kemp, B.E., Rodriguez, H., Chen, E.Y., Hudson, P.J., Martin, T.J. and Wood, W.I. (1987). A parathyroid hormone-related protein implicated in malignant hypercalcemia: cloning and expression. Science *237*, 893–896.

Trupp, M., Ryden, M., Jornvall, H., Funakoshi, H., Timmusk, T., Arenas, E., and Ibanez, C.F. (1995). Peripheral expression and biological activities of GDNF, a new neurotrophic factor for avian and mammalian peripheral neurons. J. Cell Biol. *130*, 137–148.

Tsuchida, T., Ensini, M., Morton, S.B., Baldassare, M., Edlund, T., Jessell, T.M., and Pfaff, S.L. (1994). Topographic organization of embryonic motor neurons defined by expression of LIM homeobox genes. Cell *79*, 957–970.

Vejsada, R., Sagot, Y., and Kato, A.C. (1995). Quantitative comparison of the transient rescue effects of neurotrophic factors on axotomised motoneurons *in vivo*. Eur. J. Neurosci. *7*, 108–115.

Wong, V., Pearsall, D., Arriaga, R., Ip, N.Y., Stahl, N., and Lindsay, R.M. (1995). Binding characteristics of ciliary neurotrophic factor to sympathetic neurons and neuronal cell lines. J. Biol. Chem. *270*, 313–318.

Yan, Q., and Johnson, E.J. (1988). An immunohistochemical study of the nerve growth factor receptor in developing rats. J. Neurosci. *8*, 3481–3498.

Yan, Q., Elliott, J., and Snider, W.D. (1992). Brain-derived neurotrophic factor rescues spinal motor neurons from axotomy-induced cell death. Nature *360*, 753–755.

Yan, Q., Matheson, C., and Lopez, O.T. (1995). *In vivo* neurotrophic effects of GDNF on neonatal and adult facial motor neurons. Nature *373*, 341–344.

Yoshida, K., Taga, T., Saito, M., Suematsu, S., Kumanogoh, A., Tanaka, T., Fujiwara, H., Hirata, M., Yamagami, T., Nakahata, T., Hirabayashi, T., Yoneda, Y., Tanaka, K., Wang, W.Z., Mori, C., Shiota, K., Yoshida, N., and Kishimoto, T. (1996). Targeted disruption of gp130, a common signal transducer for the interleukin 6 family of cytokines, leads to myocardial and hematological disorders. Proc. Natl. Acad. Sci. USA *93*, 407–411.